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Rapid Pyrosequencing and Fatty Acid Analysis for Characterization of *Bacillus cereus* Isolates from Food

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Summary

In this study rapid pyrosequencing was used for identification and fatty acid analysis of enterotoxic and non-enterotoxic *Bacillus cereus*. Presumptive *B. cereus* strains were isolated from ready-to-eat foods on mannitol-egg yolk-polymyxin agar and the identification was made based on the characteristics of *B. cereus* sp. Pyrosequencing was carried out on amplicons derived from 3 different 16S gene regions of rDNA using commercial reagent kits. All 30 presumptive *Bacillus cereus* isolates were identified as *B. cereus*. At least two sequencing reads of 3 different 16S rDNR gene regions were specific for identification of individual *B. cereus* isolates. Among *B. cereus* isolates, 53.3 % were found to be enterotoxic (determined by BCET-RPLA immunoassay kit). Fatty acids produced by more than 50 % of the investigated *B. cereus* were assumed as typical for these bacteria. The analyzed *B. cereus* produced a total of 25 typical fatty acids. The strains were highly homogeneous with dominant C4:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C22:6n3 fatty acids. Enterotoxic *B. cereus* was differentiated from the non-enterotoxic by significantly lower amounts of C18:0 and the absence of C18:4 fatty acid. Non-enterotoxic *B. cereus* did not produce C21:0, C17:0, C17:1, C20:0 and C15:1 fatty acids. The observed differences of individual fatty acid amounts and similar composition of fatty acids within all investigated *B. cereus* strains allowed the characterization of these bacteria isolated from ready-to-eat foods.

Key words: ready-to-eat food, *Bacillus cereus*, pyrosequencing, enterotoxins, fatty acids

Introduction

In both of its forms, spore and vegetative, *Bacillus cereus* is a common inhabitant of many different environments and can easily contaminate food. If contaminated food is not sufficiently cooled after cooking and there is an extended time between its preparation and consumption, then the surviving heat-resistant spores can germinate, enabling the organism to multiply and produce toxins. Rice, pasta, meat, poultry, vegetable dishes, various soups, puddings and sauces have all been implicated in *B. cereus* food poisoning (*1–3*).

The *B. cereus* group consists of six recognized species: *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* (*4–6*). Foodborne pathogen *B. cereus* causes diarrhoea, vomiting and produces several toxins that contribute to its virulence (*7*). However, some of the *B. cereus* virulence factors such as genes encoding enterotoxins may also be found in strains of *B. anthracis* and *B. thuringiensis* (*8*,*9*). Many traditional microbiological identification techniques have been applied to identify the *Bacillus* spp. (*10*,*11*), but accurate identification of these bacteria is difficult in many cases because they share many morphological and biochemical properties (*12*). Fatty acid methyl ester profiles have long been recognized as useful biochemical markers for bacterial classification and characterization. The analysis of fatty acids of investigated bacteria, which share many properties, must be combined with genotypic methods. DNA sequencing has been shown to be useful for identification and subtyping of a variety of bacteria (*13*,*14*). The method is optimal for sequencing of short sequences

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(typically 20–30 bases of a DNA) rapidly and in a semi-automated format (*15*). Its advantages in providing rapid detection, typing and identification of bacteria over the conventional PCR and sequencing methods have been demonstrated in studies for various bacteria (*16–18*).

The aims of the study are to assess the potential use of rapid pyrosequencing for identification of *Bacillus cereus* isolated from ready-to-eat foods and to determine differences in fatty acid composition between the enterotoxic and non-enterotoxic *B. cereus*.

Materials and Methods

Bacterial isolates

All 30 cultures of presumptive *B. cereus* investigated were isolated from ready-to-eat foods on mannitol-egg yolk-polymyxin agar (Oxoid, Basingstoke, UK). Identification of these isolates as *B. cereus* spp. was made based on their growth characteristics, biochemical testing (*19*), and the ability to grow on selective *B. cereus* chromogenic medium (BACARA; AES Chemunex, Bruchsal, Germany).

DNA extraction and amplification

Isolates cultured on nutrient slant agar (Liofilchem, Roseto Degli Abruzzi, Italy) at 30 °C for 24 h were suspended in distilled water. The obtained suspension was centrifuged at 900×*g* for 15 min. Bacterial DNA was extracted automatically with QIAcube using QiAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated DNA was frozen at –20 °C and maintained until the PCR reaction.

DNA amplification was performed using a BlackLight Sepsis Kit (2B BlackBio, Madrid, Spain), which includes 3 different amplification reactions, one of each amplifies a different region of the 16S rDNR gene (V1, V2 or V3). PCR reaction tubes from BlackLight Sepsis Kit containing BlackZyme Ultrapure DNA polymerase, dNTPs, $MgCl_2$ and oligonucleotides were supplemented with 45 μL of sterile distilled water and 2 μL of isolated DNR for each sample. PCR amplification of the target DNA was performed on a GTQ-Cycler 96 thermocycler (Hain Lifescience, Nehren, Germany). PCR protocol was as follows: initial denaturation step for 5 min at 95 °C, amplification reactions were performed with 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C and final extension step for 2 min at 72 °C.

DNA pyrosequencing

A volume of 20 μL of PCR product was mixed with 18 μL of sterile water, 40 μL of binding buffer (Qiagen) and 2 μL of streptavidin Sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden) in reaction tubes, and incubated for 20 min in a thermo shaker (Biosan, Riga, Latvia) at 1400 rpm. The immobilized biotinylated PCR product/streptavidin Sepharose complex was captured using a PyroMark Q24 Vacuum Prep Workstation (Qiagen) following the manufacturer's instructions. A volume of 2 μL of sequencing primer from BlackLight Sepsis Kit was annealed in 22 μL of annealing buffer (Qiagen) by heating at 80 °C for 2 min, followed by slow cooling to room temperature. For automatic pyrosequencing by PyroMark Q24, a Gold24 reagent kit (Qiagen) was used according to the manufacturer's instructions.

The resulting sequences were analyzed using PyroMark Q24 software (Qiagen). The sequences defined by the pyrograms as of acceptable quality were used for the identification of isolates at the GenBank (*20*). A sequence homologue match reaching 100 % was considered as an acceptable organism identification in this study. Isolates were identified as *B. cereus* if at least two amplified regions (V1, V2 or V3) of the 16S rDNR gene matched the same species in GenBank. A reference *B. cereus* ATCC 11778 strain was used for the comparison of pyrosequencing data.

Enterotoxin detection

Enterotoxins were detected using the BCET-RPLA (Oxoid, Basingstoke, UK) commercial immunoassay kit, which detects *hblC* gene in enrichment cultures. The kit was used on each purified isolate according to the manufacturer's instructions.

Fatty acid analysis

Fatty acids were separated using a gas chromatograph equipped with flame ionization detector. The bacteria for the analysis were grown on tryptic soy agar (Liofilchem) for 24 h at 30 °C. Approximately 150–200 mg of colony material was transferred by a loop from the third quadrant of the streak plate to glass tubes according to Sasser (*21*). Fatty acids were extracted using 2 mL of *n*-hexane and were methylated with KOH/methanol solution yielding methyl esters according to the LST EN ISO 12966-2:2011 (*22*). Fatty acid methyl esters were analyzed with a Shimadzu GC-2010 (Shimadzu, Kyoto, Japan) gas chromatograph using 120-m column BPX70 according to LST EN ISO 15304:2003 (*23*). A Supelco 37 Component FAME Mix reagent kit (Supelco Analytical, Thermo Scientific, Bellefonte, PA, USA) was used for the identification of fatty acids. Analysis conditions were as follows: column temperature was 60 °C for 2 min, then increased to 230 °C at 13 °C/min and held for 55 min; injector temperature was 250 °C and detector temperature 270 °C; nitrogen was used as the carrier gas.

Statistical analysis

Fatty acid analysis was performed by Student's *t*-test of independent samples using SPSS v. 16.0 software (IBM, Armonk, NY, USA). Mean values with their standard deviations were calculated from 3 replicates generated for the same *B. cereus* culture.

Results

The identification of 30 presumptive *Bacillus cereus* isolated from ready-to-eat foods confirmed that all isolates were indeed *B. cereus* spp. Sixteen of these isolates were positive for diarrhoeal type enterotoxin production (Table 1). All isolates were identified as these species by pyrosequencing as well.

The pyrosequencing using 3 different amplification reactions for each isolate produced 35 to 44 base pair nucleotide sequences. Not all amplified regions of the

Table 1. Distribution of enterotoxic *B. cereus* within investigated foods

Food	No. of isolated *B. cereus* strains	No. of enterotoxic *B. cereus*
salads with treated components	14	9
potato dishes	3	1
sweet dishes	4	0
meat dishes	2	2
floury dishes	3	1
garnish	4	3

16S rDNR gene (V1, V2 or V3) were specific for identification of *B. cereus* because in 27 pyrosequencing reactions only 2 sequences were generated. Within the investigated isolates, 8 sequence variations were detected in the V1 region, 2 variations in V2 and only 1 variation was observed in the V3 region. The obtained sequences for *B. cereus* identification are shown in Table 2. These sequences had the highest query coverage, lowest E value and highest maximum identity scores in GenBank.

In 25 pyrosequencing reactions V1 was not specific for 3 reactions, V2 for 13 reactions and V3 for 6 reactions. The obtained sequences of these regions of 16S rDNR gene did not find any significant matches in the database or they did not occur in general. Only 3 *B. cereus* isolates were identified by all V1, V2 and V3 reactions. An example of the pyrogram obtained from a foodborne *B. cereus* is shown in Fig. 1.

The sequences generated for reference *B. cereus* ATCC 11778 strain were the following: V2: GTGGCTTTCTGGTTAGGTACCGTCCAAGGTGGCCAGCCG, and V3: GTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTA. Region V1 of 16S rDNR gene of reference *B. cereus* was not successfully pyrosequenced, while V2 and V3 regions of this gene were the same as of foodborne *B. cereus*.

Fatty acids produced by more than 50 % of the investigated *B. cereus* were assumed as typical for these bacteria. The analyzed *B. cereus* produced a total of 25 typical fatty acids, 17 of which made up less than 2 % of the total content. The composition of fatty acids within enterotoxic and non-enterotoxic *B. cereus* is shown in Table 3.

Fatty acids C15:1, C17:0, C17:1, C20:0 and C21:0 were not determined within non-enterotoxic *B. cereus*, while C18:4 fatty acid was not determined within enterotoxic *B. cereus*. Butyric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic and docosahexaenoic fatty acids were the dominant fatty acids in all *B. cereus* strains. The amounts

Table 2. The obtained sequences for *B. cereus* isolates by pyrosequencing with V1, V2 and V3 primers

Primer	Sequences	GenBank (BLASTN) accession
V1	AACTTCATAAGAGCAAGCTCTTAATCCATTCGCTCCGA	DQ339675.1
	AACTCTTAGAGCAAGCTCTCAATCCATTCCGTCCTTCCGA	JN252060.1
	AACTTCTTAGAGCAAGCTCTCAATCCATTCGCTCCGA	JN252060.1, JN252098.1
	AACTTCATAAGAGCAAGCTCTTAATCCATTCGCTCCGA	DQ339675.1
	AACTTCATAAGAGCAAGCTCTTAATCCATTTCGCTCCGA	GU930747.1
	AACTTCTTAGAGCAAGCTCTCAATCCATTCGCTCGA	JN252060.1
	AACTTCATAAGAGCAAGCTCTTAATTCCAATTTCCGTCCTCCGA	HQ670527.1
V2	GTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCACGC	CP003187.1
	GTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGC	JN252098.1
V3	GTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTA	JN252089.1, JN187086.1

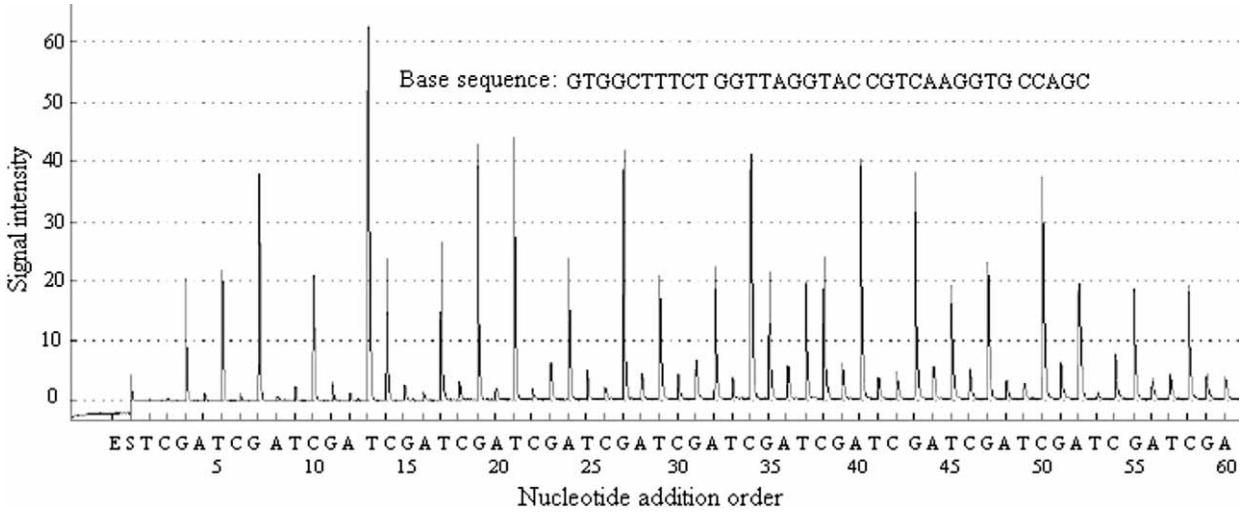

Fig. 1. Pyrogram of a foodborne *Bacillus cereus*

Table 3. Fatty acid composition of *Bacillus cereus* isolates

Fatty acid	*B. cereus*		p-value
	Entero-toxic	Non-entero-toxic	
butyric (C4:0)	15.8±1.7	14.7±2.3	>0.05
caprylic (C8:0)	0.2±0.1	0.3±0.1	>0.05
capric (C10:0)	0.5±0.1	0.7±0.2	>0.05
undecanoic (C11:0)	0.4±0.1	0.5±0.1	>0.05
lauric (C12:0)	0.8±0.1	1.0±0.1	>0.05
tridecanoic (C13:0)	1.1±0.3	1.1±0.3	>0.05
myristic (C14:0)	3.2±0.4	4.2±0.4	>0.05
pentadecanoic (C15:0)	0.7±0.3	0.5±0.1	>0.05
cis-10-pentadecenoic (C15:1)	1.2±0.3	–	<0.05
palmitic (C16:0)	12.5±1.6	14.6±1.4	>0.05
palmitoleic (C16:1)	2.3±0.3	2.5±0.4	>0.05
heptadecanoic (C17:0)	1.3±0.5	–	<0.05
cis-10-heptadecanoic (C17:1)	1.2±0.4	–	<0.05
stearic (C18:0)	4.8±0.5	6.9±0.5	<0.05
oleic (C18:1)	19.8±3.4	20.2±2.4	>0.05
elaidic (C18:1 trans)	0.8±0.2	0.7±0.2	>0.05
α-linolenic (C18:3)	1.3±0.4	0.9±0.2	>0.05
γ-linolenic (C18:3)	1.2±0.2	1.0±0.2	>0.05
linoleic (C18:2)	6.7±1.1	6.8±1.1	>0.05
octadecatetraenoic (C18:4)	–	1.3±0.2	<0.05
arachidic (C20:0)	0.5±0.2	–	<0.05
eicosatrienoic (C20:3n3)	1.4±0.3	0.7±0.2	>0.05
eicosapentaenoic (C20:5n3)	0.4±0.1	0.5±0.1	>0.05
heneicosanoic (C21:0)	0.2±0.1	–	<0.05
docosahexaenoic (C22:6n3)	13.2±2.6	12.1±2.1	>0.05

$p<0.05$ was considered significant

of dominant fatty acids did not significantly differ among pathogenic and non-pathogenic *B. cereus*, *i.e.* the isolates from ready-to-eat foods were highly homogeneous regarding the dominant fatty acids. The amounts of C18:0 produced in non-enterotoxic *B. cereus* were significantly higher ($p<0.05$) than in enterotoxic *B. cereus* cultures.

No significant differences were determined among total amounts of saturated, monounsaturated, polyunsaturated, *trans* fatty acids and omega-3 fatty acids produced by enterotoxic and non-enterotoxic *B. cereus*. All *B. cereus* strains exhibited high proportion of saturated fatty acids (48.54 %) and the low proportion of *trans* fatty acids (1.32 %). The total amounts of monounsaturated and polyunsaturated fatty acids produced in *B. cereus* were 22.82 and 25.22 %, respectively.

To determine the fatty acid profile of the other representative species from *Bacillus* genus, the reference strain of *B. subtilis* ATCC 6633 was chosen. Comparison of foodborne *B. cereus* strains and the reference strain of *B. subtilis* showed some differences in the production of fatty acids. *B. subtilis* was characterized by production of higher amount of palmitic (C16:0; 20.4 %) acid, the presence of miristoleic (C14:1; 3.7 %) and hexadecanoic (C16:2; 2.6 %) acids, and the absence of heptadecanoic (C17:0) and *cis*-10-heptadecanoic (C17:1) acids. The absence of C17:0 and C17:1 fatty acids was typical for non-enterotoxic *B. cereus* isolates, while enterotoxic *B. cereus* produced these fatty acids.

Discussion

Pyrosequencing was succesfully applied for the identification of many bacteria (*13*,*24*,*25*), but there is a lack of studies about its application using rapid methods for detection of foodborne *B. cereus*. Pyrosequencing combines the advantages of the PCR amplification and sequencing technique for bacterial identification (*24*). In this study, the obtained data about the identification of foodborne *B. cereus* is useful for rapid detection of these pathogenic bacteria. The BlackLight Sepsis Kit containing necessary primers and sequencing oligonucleotides has been designed to fully characterize sepsis from different samples no matter where bacteria gain entry to the body, causing tissue contamination. This kit has been tested with samples from different origins including blood, haemocultures, liver, or gastrointestinal tract, according to the manufacturer's instructions, but we were unable to find any data about its application for bacteria isolated from food. Literature data about rapid pyrosequencing assay for distinguishing *B. anthracis* from the *B. cereus* group were found (*26*). The nucleotide at the third position in the pyrosequence was represented by a conserved T nucleotide in all *B. anthracis* strains and a conserved A nucleotide in all non-*B. anthracis* strains in pyrosequencing data. A specific sequence CTTCTGGTG was determined for *B. anthracis*, while other *Bacillus* spp., *i.e. B. thuringiensis*, *B. mycoides* and *B. cereus* in many cases had a part of CTATTGTAGTAAT nucleotide sequence. In our study, different nucleotide sequences were obtained and the sequence AACTT was determined in the investigated isolates in V1 region of 16S DNA. These differences could be explained by the use of a reagent kit with the universal primers in this study; however, the pyrograms for *B. cereus* obtained in this study did not differ from the pyrograms presented by others researches (*26*,*27*). By comparing pyrosequencing data obtained in this study, the determined primary structures of at least two sequencing reads of 16S rDNR gene of foodborne *B. cereus* enabled the identification of *B. cereus*. Moreover, V2 and V3 sequencing reads of 16S rDNR gene of *B. cereus* ATCC 11778 were successfully pyrosequenced. Rapid pyrosequencing using reagent kits eliminates many problems associated with labelled nucleotides, gel electrophoresis, and PCR product purification. It provides fast identification of *B. cereus* with a minimum of manual work. Other researchers (*28*) described the use of real-time DNA sequence analysis of *Helicobacter pylori* 16S rRNA gene fragments by pyrosequencing based on DNA sequence heterogeneity within the variable regions V1 and V3. Automated system enabled a rapid determination of 20–30 nucleotides of target sequences and the authors concluded that DNA sequence variations occured in the 16S rDNA variable regions V1 and V3 of *H. pylori* providing a consistent system for subtyping.

The identification of bacteria using traditional microbiological methods usually takes up to a few days. In addition, toxin identification requires purification of the

toxin before analysis. For these reasons, we analyzed fatty acid compositions of *B. cereus* to explore the possibility of a faster differentiation of enterotoxic and non-enterotoxic *B. cereus*. The types and relative abundances of fatty acids produced within a cell are largely determined by an organism's genotype and can be used for identification of different species (*29*) and strains (*30,31*). Commercial systems streamline fatty acid extraction and detection procedures (*21*), but our previous work showed that *B. cereus* isolated from dried milk products can be differentiated by fatty acid compositions using a daily chromatographic method (*32*). The tested foodborne *B. cereus* strains were characterized by butyric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic and docosahexaenoic fatty acids. The amounts of these fatty acids in *B. cereus* varied from 2.30 to 20.22 % of total content. Enterotoxic *B. cereus* isolates produced significantly lower amount of stearic fatty acid. In literature, stearic, myristic and lauric fatty acids are common although lesser constituents of microbial lipids (*33*). In our research, however, the lowest amount of palmitoleic fatty acid (2.4 % of a total content) was determined in *B. cereus* strains. On the other hand, C20-28 fatty acids are encountered only in a few species, and the tested *B. cereus* strains produced 12.64 % of docosahexaenoic (C22:6n3) fatty acid and trace amounts of C20:0, C20:3n3, C20:5n3 and C21:0 fatty acids. Palmitic acid occurs more frequently and usually in larger amounts than any other saturated acid found in bacteria (*33*). This was confirmed with the tested *B. cereus*. Monounsaturated oleic fatty acid occurred in larger amount (20.01 %) in all investigated *B. cereus*. Similar result were obtained by other scientists (*34*), who determined oleic fatty acid (7.90 % of a total content) as biomarker for *B. cereus* T-strain spores grown on Columbia agar supplemented with sheep blood.

Fatty acid analysis was applied for characterization of *B. licheniformis*, *B. pumilus* and *B. subtilis* isolated from aerobic waste treatment bioreactors. All investigated strains were characterized by the predominance of i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0 fatty acids, and a15:0 was the major fatty acid in all strains. *B. licheniformis* strains exhibited the highest proportion of branched fatty acids (85.32 %) and the lowest proportion of saturated fatty acids (11.94 %), whilst *B. pumilus* strains displayed the highest proportion of saturated fatty acids, due to the highest percentage of C16:0 produced by this species (16.3–22.1 %) (*35*). All foodborne *B. cereus* isolates exhibited the highest proportion of saturated fatty acids (48.54 %) and the lowest proportion of *trans*-fatty acids (1.32 %), while the major oleic C18:1 fatty acid occurred in both enterotoxic and non-enterotoxic *B. cereus*. The data of fatty acid analysis of the reference *B. subtilis* strain conformed with the data presented in the references. It has been found that only two strains of *B. subtilis* (from 6 investigated) isolated from aerobic waste treatment bioreactors produced C17:0, and all strains did not produce C17:1 (*35*).

The BCET-RPLA kit is a simple and highly sensitive immunological method that detects *B. cereus* enterotoxin. The disadvantage of this test is that it reacts with a toxin from *B. thuringiensis*, which is closely related to *B. cereus* (*36*). Therefore, the determination of enterotoxin in presumptive *B. cereus* can give false results (*37,38*) if this test is used for identification of *B. cereus*. The observed differences of individual fatty acid amounts and the similar composition of fatty acids within all investigated *B. cereus* strains allowed the differentiation of these bacteria isolated from ready-to-eat foods. *B. cereus* differentiation by fatty acid analysis is cheaper than using BCET--RPLA kit. The duration of fatty acid analysis is shorter, too, because the analysis of pure cultures can be performed immediately, which is contrary to the BCET--RPLA test, where isolated *B. cereus* must be incubated in a brain-heart infusion for 24 h before analysis.

The rapid detection of pathogens in food is critical for ensuring the safety of consumers. Rapid identification by pyrosequencing and confirmation of pathogenic *B. cereus* by fatty acid analysis could be used as additional methods for the characterization of foodborne *B. cereus*.

Conclusions

The results presented here show that rapid pyrosequencing includes minimum manual work (less than 4 h), which makes it suitable for *B. cereus* identification. The results showing the variation of fatty acid content in *B. cereus* isolates enable the differentiation of these bacteria into enterotoxic and non-enterotoxic strains. The obtained results are important for rapid differentiation of pathogenic *B. cereus* that can cause food poisoning.

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